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(54) Title: METHOD AND REAGENT FOR DETECTION OF ENDOTOXINES OR β-1,3 GLUCANES FROM FUNGUS OR BACTERIA

(57) Abstract

Method of detecting a fungal or bacterial infection by contacting a sample to be tested with (A) a blood cell lysate from a crustacean or an insect and (B) a detector substance in the form of a peptide compound having a specific terminal group, which through the enzymatic action of a blood cell lysate obtained from such an animal and activated by a bacterium or a fungus may be cleaved off to form a physically or chemically detectable compound. The invention also relates to a reagent or a reagent kit comprising said blood cell lysate and detector substance.

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METHOD AND REAGENT FOR DETECTION OF ENDOTOXINES OR β-1,3-GLUCANES FROM FUNGUS OR BACTERIA

The present invention relates to a method and a reagent for detecting bacterial and fungal infections with a high sensitivity.

Various methods for rapidly detecting bacterial infections through Gram-negative bacteria with a relatively high sensitivity are based upon the reaction of a lysate of amebocytes or "blood cells" from horseshoe crabs. The lysate reacts to bacterial endotoxins, which are lipopolysaccharides, such that a gel is formed. This gel phenomenon, which is a defense reaction of the horseshoe crab, is elicited by the lipopolysaccharides activating a serine protease, which in turn converts coagulogen into coagulin. The previous determination methods are based, on one hand, on the gel reaction itself, such as measurement of the gel formation rate, turbidity changes, etc., and, on the other hand, upon the activated serine protease. The last mentioned method, which is the definitely most sensitive one and permits detection of concentrations as low as 10^{-6} - 10^{-9} mg/ml, utilizes the capability of the serine protease to enzymatically hydrolyze certain synthetically prepared polypeptides, such that specific end groups are cleaved off and thereby form compounds which may be detected colorimetrically or fluorometrically. This method is described in, for example, the German Offenlegungsschrift 2,740,323. A commercial reagent, marketed by Kabi Peptide Research Ltd., Mölndal, Sweden, contains a synthetic peptide having a terminal p-nitroanilide group and a lysate from the American horseshoe crab or Limulus polyphemus. On addition of bacterial lipopolysaccharides the serine protease of the lysate is activated and the yellow colour of the p-nitroaniline released thereby may be read colorimetrically.

While the above described reaction applies to blood cell lysates from all horseshoe crab species, there is only a small number of living species of this early, almost fossile marine animal. Among those the above horseshoe crab, Limulus polyphemus, which exists along the American Atlantic coast, and the Japanese horseshoe crab, Tachypleus tridentatus, may be mentioned. These animals, which thus are relatively rare in the oceans, are also becoming depleted, and this is particularly the case for Limulus polyphemus which is the one that has so far been most utilized commercially for the above mentioned test method. Since the horseshoe crab cannot be cultured in an aquaculture, a shortage of such lysate may be expected in the future.

Till now there has, however, been no substitute for the blood cell lysate of the horseshoe crab, the horseshoe crab being regarded as an old and primitive

odd branch of the articulates or arthropods with a unique blood system which in several respects differs substantially from those of other arthropods. The above mentioned endotoxin reaction has therefore been believed to be limited to this specific animal species. Thus, for example, the horseshoe crab only has one type of blood cells, while the other arthropods have three to nine distinct blood cell types depending on class and species. A more important difference in this connection is, however, that the coagulogen of the horseshoe crab is in the blood cells, while in other arthropods it has been shown to be included in the plasma, where the main coagulation reaction also takes place. Both plasma and blood cells would therefore be required for a coagulation activation in the latter case, which thus would make the preparation of a fairly stable activable preparation like that from the horseshoe crab impossible.

According to the invention it has, however, been found, that coagulogen and at least one serine protease are included in the blood cells of the arthropods crustaceans and insects and are involved in the activation of a coagulation-like reaction in a similar way as in the horseshoe crab, and that therefore both bacterial and fungal infections may be determined qualitatively as well as quantitatively using a blood cell or hemocyte lysate from these animals. A lysate from these arthropods is activated quantitatively by lipopolysaccharides, or LPS, i.e. bacterial endotoxin, as well as by another type of carbohydrates, viz. β-1,3-glucans, which are part of the cell walls of essentially all fungi. While the biochemical mechanism is not completely known, the β-1,3-glucans very specifically activate a serine protease which, in addition to converting coagulogen into coagulin, converts prophenoloxidase into the active enzyme phenoloxidase.

According to the invention a fungal or bacterial infection may thus rapidly and easily be detected by, in a per se known manner, contacting a sample to be examined with, on one hand, a blood cell lysate from crustaceans or insects, or an activable serine protease or proteases isolated therefrom, and, on the other hand, a detector substance which through the enzymatic action by such a blood cell lysate activated by a bacterial or fungal extract may form a physically or chemically detectable compound, and determining the latter. A corresponding reagent or reagent kit for detecting or determining fungal or bacterial infections therefore comprises, on one hand, a blood cell lysate from crustaceans or insects and, on the other hand, such a detector substance.

Although generally blood cell lysates from all crustaceans and insects could be used according to the invention, among the crustaceans (Crustacea) the

ten-footed crustaceans or the so-called decapods are preferred. Fresh-water decapods as well as marine decapods may be used. As examples of fresh-water crayfish may be mentioned Astacus astacus (river crayfish), Pacifastacus leniusculus (signal crayfish), Astacus pallipes, Orconectes limosus, Astacus leptodactylus, Cambarus affinis (North-American crayfish), Procambarus clarkii. Among suitable marine decapods Cancer pagurus (common or edible crab), Carcinus maenas (shore crab), Homarus vulgaris (lobster), Palinurus vulgaris (spiny lobster), Nephrops norvegicus (Norwegian lobster), Callinectes sapidus may be mentioned. Among these crustaceans several are directly suited for culturing in an aquaculture, e.g. river crayfish and signal crayfish, but also lobster and crab, and these are therefore preferred for the purposes of the invention.

Among insects particularly those of Orthoptera and Lepidoptera (butterflies) may be mentioned. Examples of the first mentioned order are Schistocerca gregaria (desert locust) and Locusta migratoria (common locust). Among butterflies Galleria melonella (wax-moth), Hyalphora cecropia and Bombyx mori (silk-moth) may be mentioned. Although insects probably would not be of the same interest for lysate production as crustaceans, the culture of, for example, silk-moth for this purpose might very well be contemplated.

Due to the fact that blood cell lysates from, e.g., the above mentioned crustaceans may be used, a simple and continuous source of blood cell lysates having a very uniform quality is assured, since hemolymph from, e.g., crayfish may be collected during the major part of the year. The hemolymph of the horseshoe crab, on the other hand, may only be collected during a very limited period of time.

As mentioned above the coagulation process for hemolymph from crustaceans and insects involves activation of at least one serine protease. Due to the presence of such an active serine protease essentially the same type of peptide compounds, which have been used or suggested for the previously known Limulus lysate process, may be used. Thus, the invention comprises a method and a reagent, wherein the detector substance, i.e. the substance to be affected by the activated lysate, is a peptide compound of the formula

$$R_1\text{-}X\text{-}Arg\text{-}R_2$$

wherein R_1 is a peptide moiety protected at the N-terminal and having at least two amino acid units, X is Gly or Ala, preferably Gly, and R_2 is a residue attached to the C-terminal of the arginine residue represented by Arg through an acide amide and/or ester linkage, and which in the presence of the blood cell lysate

activated by bacteria or fungi may be hydrolyzed enzymatically into R_2H, and/or a mineral acid salt thereof. The residue R_2 is further such that the compound R_2H formed may be detected physically or chemically, e.g. colorimetrically or spectrophotometrically, and is preferably a fluorogenic or a chromogenic compound. Preferably, the group R_2 is derived from p-nitroanilide, 5-nitro-α-naphtylamide, β-naphtylamide, α-naphtyl ester, β-naphtyl ester, indoxyl ester, N-methylindoxyl ester, (4-methyl)umbelliferyl ester and/or resorfin ester, the corresponding compounds R_2H being p-nitroaniline, 5-nitro-α-naphtylamine, β-naphtylamine, α-naphtol, β-naphtol, indoxyl, N-methyl-indoxyl, 4-methyl-umbelliferon and resorfin. Of these the first two compounds are chromogenic, while the other are fluorescent compounds.

Suitable protecting groups for R_1 are, for example, benzoyl, acetyl, carbobensoxy, tert-butoxycarbonyl and p-toluenesulfonyl.

Particularly suitable peptide derivatives for use in this aspect of the invention are

Bz-Ile-Glu-Gly-Arg-PNA.HCl
Bz-Ile-Glu(γ-piperidyl)-Gly-Arg-PNA.HCl
Bz-Ile-Glu(O-Et)-Gly-Arg-PNA.HCl
Bz-Ile-Glu(O-i-Pr)-Gly-Arg-PNA.HCl
Bz-Ile-Ser-Gly-Arg-PNA.HCl
Bz-Ile-Glu(O-Me)-Gly-Arg-PNA.HCl

wherein Bz = benzoyl, Ac = acetyl, Me = methyl, Et = ethyl, i-Pr = isopropyl and -PNA = p-nitroanilide, and the amino acids are given with the IUPAC abbreviations.

Some of the peptide compounds are commercially available while the others may be prepared with known methods.

In other respects the process may be effected as is described for the Limulus lysate method in the above mentioned German Offenlegungsschrift 2,740,323.

A hemocyte lysate, i.e. a blood cell lysate, from crustaceans and insects according to the invention may be prepared essentially in the same way as the well known Limulus lysate from the horseshoe crab. This method is well described previously in the literature and need therefore not be described in any detail herein. Thus, a partially purified hemocyte lysate from, for example, crustaceans is prepared by first collecting blood from the animal, care being taken to avoid contamination of the blood. It is to be noted that it is not necessary to kill a crustacean which can be cultured in an aquaculture, such as

crayfish, signal crayfish, etc., when collecting the blood, but blood may be collected from the same animal several times at regular intervals as with a human blood donor. This is, of course, a great advantage, since the recovery of blood for hemocyte lysate production according to the invention may then be combined with crayfish culture for food purposes. The hemocytes or blood cells are then isolated through centrifuging and washing in conventional manner. After that they are homogenized in a buffer with a high calcium ion concentration, centrifuged at about 70,000 g and the supernatant recovered. The lysate obtained is more stable than the present commercial lysates from horseshoe crabs and will remain stable for at least 24 hours. Preferably, however, the supernatant obtained is freeze-dried to be diluted with water or a suitable buffer (pH ~ 8) at the time of use. The solution may optionally be stabilized with 0.5 - 1.5M NaCl, whereby a stability of several days may be achieved. Also a solution of the freeze-dried lysate is stable for at least 5 hours. To increase the freeze-drying stability, e.g., bovine serum albumin and/or glycine may be added.

A reagent or reagent kit according to the invention for detecting fungal and bacterial infections may comprise a blood cell lysate prepared as above and a detector substance according to the previous definition. Preferably, the lysate and the detector substance are in powder form. When using it for testing, for example, an extract having a suspected fungal or bacterial infection, the test reagent is dissolved in a suitable buffer (pH ~ 8), and then the extract to be tested is added. The reagent solution is then studied, for example, spectrophotometrically or colorimetrically depending on the detector substance used.

The method and the reagent of the invention are, in comparison with the horseshoe crab lysate known for endotoxin determination, also extremely sensitive for detecting fungal infections. All fungi containing β-1,3-glucans in the cell walls thereof may be detected, which applies to all existing fungi with few exceptions. To rapidly be able to detect fungal infections in accordance with the invention is of great value. At present, fungal skin infections on humans and animals are cultured, which takes about 2 weeks. Also, mould infections in food, so-called mycotoxins, are becoming a rapidly increasing problem.

The sensitivities hitherto obtained with the use of lysates from crustaceans according to the invention are about 10^{-8} - 10^{-9} g/ml to endotoxins (bacterial infection) and about 10^{-10} g/ml to β-1,3-glucans (fungal infection). The sensitivity to endotoxins may probably be increased further by eliminating inhibitors of the endotoxin activation which have been shown to be included in

the lysate, for example analogously to what is now done with the commercial horseshoe crab lysates.

The blood cell lysates may be rendered endotoxin specific by adding an excess of β-1,3-glucans. In a corresponding manner β-1,3-glucan specificity may be achieved by adding anti-endotoxin factors, produced in pure form from crayfish lysate, which will then prevent the endotoxin activation of the crayfish lysate. Alternatively, high contents of endotoxin (LPS) may be added.

Quantitative determinations of endotoxins and β-1,3-glucans, respectively, by means of the inventive process may be effected in a manner known per se, e.g. by providing a standard or calibration curve.

The invention will now be described in more detail by means of some specific examples, which, however, are not limiting to the invention in any way.

Example 1

Preparation of a hemocyte lysate

Hemocytes from crayfish, Astacus astacus, were collected as described in Söderhäll, K., Häll, L., Unestam, T., and Nyhlén, L. (1979) J. Invertebr. Pathol. 34, 285-294, except that 0.1M sodium citrate was excluded. The hemocytes were homogenized in 10 mM sodium cacodylate buffer, pH 7.0, with 100 mM $CaCl_2$ and the homogenate was then centrifuged for 20 minutes at 70,000 g. The supernatant obtained, containing approximately 2 mg protein/ml, may be used either immediately or freeze-dried in 3 ml aliquots. Prior to use it is dissolved in 3 ml of distilled water to a final protein concentration of 2 mg/ml.

Example 2

β-1,3-glucan activation of crayfish hemocyte lysate

In order to test the method of the invention with regard to specific β-1,3-glucan activation of the hemocyte lysate, two volumes of the hemocyte lysate from Example 1 were mixed with one volume of β-1,3-glucans or other carbohydrates of the concentrations given in the following Table 1 for 30 minutes at 20-22°C. 100 µl of this reaction mixture was added to 600 µl of 0.1M tris-HCl-buffer, pH 8.0, and 100 µl of a 2 mM solution of the synthetic peptide Bz-Ile-Glu(γ-piperidyl)-Gly-Arg-PNA.HCl (obtained from Kabi Peptide Research Ltd., Mölndal, Sweden). After incubation for 0.5 hour at 37°C 100 µl of 50% acetic acid were added to terminate the reaction, and the released p-nitroaniline (PNA) was measured spectrophotometrically at 405 nm.

The β-1,3-glucans used were Zs (the supernatant of a 1% suspension of yeast cell walls, Zymosan, Sigma), laminaran M, laminaran G and a linear pentasaccharide composed of β-1,3-D-linked glucosyl residues. Laminaran M and

G were purified from laminaran (Sigma) by using DEAE-molybdate-Sephadex® chromatography according to Stark S., J.R. (1976) Carbohydr. Res. 47, 176-178. The linear pentasaccharide was prepared and purified as described in Söderhäll, K., and Unestam, T. (1979) Can. J. Microbiol. 25, 406-414.

The results of the enzyme activity determination (serine protease) showing the specific β-1,3-glucan activation appears from Table 1 below.

Table 1

β-1,3-glucan activation of serine protease in crayfish hemocyte lysate

Glucan	Structure	Degree of polymerisation	Concentration (glucose equiv., µg/ml)	Enzyme activity (ΔA_{405}/30 min)
None	-	-	0	0.03
Zymosan supernatant	(1→3)β-D-glucan with β-1,6-linkages	varying ($2 \cdot 10^4 - 10^6$)	60	0.85
Laminaran M	(1→3)β-D-glucan ended with mannitol	20	300	0.99
Laminaran G	(1→3)β-D-glucan ended with glucose	20	300	0.99
Laminara-pentaose	(1→3)β-D-glucan	5	300	0.95
Other carbohydrates tested*		-	300-1600	not detected

* Other carbohydrates tested were chitin, cellulose, dextran, glucose and mannitol.

Example 3

Freeze-dried hemocyte lysate from Example 1 was dissolved in 3 ml of 0.1 M tris-HCl-buffer of pH 8.0. This solution was then mixed with 1.5 ml of Zymosan supernatant according to Example 2 and incubated for 30 minutes at 20°C. From this reaction mixture 100 µl were withdrawn and added to 600 µl of 0.1 M tris-HCl (pH 8.0). 100 µl of 2 mM solutions of different synthetic peptides having chromogenic terminal groups (obtained from Kabi Peptide Research Ltd., Mölndal, Sweden) were added to this mixture, and after incubation for 1 hour at 37°C the reaction was terminated by adding 100 µl of 50% acetic acid. The released p-nitroaniline was then measured spectrophotometrically at 405 nm. The results for the different chromogenic substrates are listed in Table 2 below.

Table 2

Hydrolysis of different chromogenic substrates by preactivated crayfish hemocyte lysate

Chromogenic substrate	Enzyme activity (ΔA_{405}/30 min)
Bz-Ile-Glu-Gly-Arg-PNA.HCl	0.62
Bz-Ile-Glu(γ-piperidyl)-Gly-Arg-PNA.HCl	0.84
Bz-Ile-Glu(O-Et)-Gly-Arg-PNA.HCl	0.84
Bz-Ile-Glu(O-i-Pr)-Gly-Arg-PNA.HCl	0.92
Bz-Ile-Ser-Gly-Arg-PNA.HCl	0.90
Bz-Ile-Glu(O-Me)-Gly-Arg-PNA.HCl	0.70

Example 4

A reagent according to the invention consisting of a crayfish hemocyte lysate according to Example 1 in 0.01 M cacodylate buffer (pH 7.0) and an equal amount (about 100 µl) of Bz-Ile-Glu(γ-piperidyl)-Gly-Arg-PNA.HCl were mixed with pre-heated (5 minutes, 100°C) extracts of the following fungi: Aphanomyces astaci, Aphanomyces laevis, Aphanomyces euteiches, Saccharomyces cerevisiae, Aspergillus flavus, Penicillium viridicatum, Candida albicans, Polyporus annosus, Boletus variegatus. The solutions were then observed colorimetrically at 405 nm. For all the fungi a yellow colour was obtained indicating activation of the hemocyte lysate with associated enzymatic hydrolysis of the p-nitroanilide group to p-nitroaniline.

Example 5

A mixture of 100 µl of hemocyte lysate from Example 1, 100 µl of E. coli endotoxin (Mallinckrodt, Inc., USA) of different concentrations, 100 µl of

0.1 M tris-HCl, pH 8.0, and 100 µl of a 2 mM solution of Bz-Ile-Glu(γ-piperidyl)-Gly-Arg-PNA.HCl (from Kabi Peptide Research Ltd., Mölndal, Sweden) were incubated for 30 minutes at 37°C. 100 µl of 50% acetic acid were then added to terminate the reaction, and the released p-nitroaniline was measured spectrophotometrically at 405 nm. The absorbance changes obtained appear from Table 3:

Table 3

Endotoxin concentration g/ml	ΔA_{405}/30 min/100 µl hemocyte lysate
1×10^{-6}	0.64
1×10^{-7}	0.55
1×10^{-8}	0.18
1×10^{-9}	0.058
Control	0.030

Example 6

In analogy with Example 1 hemocyte lysates were prepared from the following crustaceans:

Pacifastacus leniusculus
Astacus pallipes
Astacus leptodactilus
Orconectes limosus
Cancer pagurus
Carcinus maenas
Nephrops norvegicus

The lysates obtained were tested for activation by β-1,3-glucans and endotoxins in analogy with the methods described in Examples 2 and 5, activation (release of p-nitroaniline) being indicated for all the lysates.

Blood cell lysates from the insects Schistocerca gregaria, Galleria melonella, Hyalphora cecropia and Bombyx mori have so far only been tested with regard to activation of phenoloxidase, which, as for the above mentioned crustacean lysates, gave positive results. Because of the great similarities bewteen the blood systems of crustaceans and insects, and since it was found in connection with the invention that phenoloxidase in turn is activated by a serine protease, also these insect lysates must be expected to be activated in testing as above.

CLAIMS

1. A method for detecting fungi and/or bacteria by detecting or determining endotoxins and β-1,3-glucans, respectively,
characterized by contacting the sample to be tested with (A) a blood cell lysate from an animal belonging to one of the arthropod classes crustaceans (Crustacea) and insects (Insecta), or an activable serine protease or proteases isolated therefrom, and (B) a detector substance in the form av a peptide compound of the formula

$$R_1 - X - Arg - R_2$$

wherein R_1 is a peptide moiety protected at the N-terminal thereof and having at least 2 amino acid units, X is Gly or Ala, and R_2 is a residue attached to the C-terminal end of the arginine residue represented by Arg through an acid amide and/or ester linkage and which in the presence of the blood cell lysate activated by endotoxin or β-1,3-glucan is capable of being enzymatically hydrolyzed to R_2H,
and/or a mineral acid salt thereof, and determining the R_2H formed, R_2H being such a compound that may be detected physically or chemically, preferably a fluorogenic or chromogenic compound.

2. A method according to claim 1,
characterized in that the blood cell lysate is a hemocyte lysate from an animal belonging to the order of decapods.

3. A method according to claim 2,
characterized in that the hemocyte lysate is derived from a fresh-water crayfish, particularly Astacus astacus or Pacifastacus leniusculus.

4. A method according to claim 2,
characterized in that said decapod is selected from Astacus astacus, Pacifastacus leniusculus, Astacus leptodactylus, Astacus pallipes, Orconectes limosus, Cambarus affines, Procambarus clarkii, Cancer pagurus, Carcinus maenas, Homarus vulgaris, Palinurus vulgaris, Nephrops norvegicus and Callinectes sapidus.

5. A method according to any one of claims 1 - 4,
characterized in that R_2 is a p-nitroanilide, 5-nitro-α-naphtylamide, β-naphtylamide, α-naphtyl ester, β-naphtyl ester, indoxyl ester, N-methylindoxyl ester, (4-methyl)umbelliferyl ester or resorfin ester group.

6. A method according to any one of claims 1 - 5, characterized in that the detector substance is selected from

Bz-Ile-Glu-Gly-Arg-PNA.HCl
Bz-Ile-Glu(γ-piperidyl)-Gly-Arg-PNA.HCl
Bz-Ile-Glu(O-Et)-Gly-Arg-PNA.HCl
Bz-Ile-Glu(O-i-Pr)-Gly-Arg-PNA.HCl
Bz-Ile-Ser-Gly-Arg-PNA.HCl
Bz-Ile-Glu(O-Me)-Gly-Arg-PNA.HCl
Ac-Ile-Glu-Ala-Arg-PNA.HCl

wherein Bz is benzoyl, Ac is acetyl and -PNA is p-nitroanilide.

7. A reagent for detecting fungi and/or bacteria by detecting or determining endotoxins and β-1,3-glucans, respectively, characterized by comprising (A) a blood cell lysate from an animal belonging to one of the arthropod classes crustaceans (Crustacea) and insects (Insecta), and (B) a detector substance as defined in claim 1, 5 or 6.

8. A reagent according to claim 7 for detecting a fungal infection, characterized by comprising at least one anti-endotoxin factor or an excess of endotoxin.

9. A reagent according to claim 7 for detecting a bacterial infection, characterized by comprising an excess of β-1,3-glucan.

10. A reagent according to any one of claims 7 - 9, characterized by comprising a blood cell lysate as defined in any one of claims 2 - 4.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE82/00430

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 12 Q 1/38 // C 12 Q 1/56

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC 3	C 12 Q 1/00,34,36,38,56
US Cl	435:4, 13, 23; 195:103.5

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [5]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US, A, 4 301 245 (LINDSAY G AND O´BEIRNE A J) 17 November 1981	1-10
A	DE, B2, 2 740 323 (SEIKAGAKU KOGYO CO LTD) 25 October 1979	1-10
A	EP, A3, 0 041 089 (DYNASCIENCES CORPORATION) 9 December 1981	1-10
E	EP, A3, 0 056 210 (PHARMINDUSTRIE) 21 July 1982 & WO, A, 82/02382	1-10
A	Patent Abstracts of Japan, Vol 5, No 102, C61, abstract of JP 56-42597, publ. 1981-04-20.	1-6
A	Chemical Abstracts Vol 94(1981), abstract No 2223q, FEBS Lett. 1980, 120(2), 217-20.	1
A	Chemical Abstracts Vol 94(1981), abstract No 151160u, Progr. Clin. Biol. Res. 1979, 29, 209-20.	1

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* Special categories of cited documents: [15]

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
1983-02-25	1983 -03- 02
International Searching Authority [1]	**Signature of Authorized Officer [20]**
Swedish Patent Office	C-O Gustafsson

Form PCT/ISA/210 (second sheet) (October 1981)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No 18
A	Chemical Abstracts Vol 95(1981), abstract No 181776n, Clin. Chim. Acta 1981, 116(1), 63-8.	1
E	Chemical Abstracts Vol 96(1982), abstract No 83106v, Dev. Comp. Immunol. 1981, 5(4), 565-73.	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02878

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :G06F 15/62
US CL :395/159,161,153
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 395/144-147,156,157,158,160; 340/716,721,723

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,885,704 (TAKAGI ET AL.) 05 December 1989, See figure 9 and column 5-6.	1-72
Y	US, A, 5,060,135 (LEVINE ET AL.) 22 October 1991, See the entire document.	1-72
A,P	US, A, 5,140,677 (FLEMING ET AL.) 18 August 1992, See the entire document.	1-72

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

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